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Biosynthesis of phytoalexin in carrot root requires ethylene action

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The role of ethylene in phytoalexin production by carrot (*Daucus carota* L.) roots was investigated using the ethylene action inhibitor 1-methylcyclopropene (MCP). Exposure of carrot roots to ethylene, UV-B irradiation, inoculation with fungal pathogens, treatment with 2,4-D or methyl jasmonate induced accumulation of the phytoalexin 6-methoxymellin (6-ME). Exposure to MCP for 4–12 h prior to the treatments completely inhibited 6-ME accumulation, indicating that 6-ME synthesis by carrot roots requires ethylene action.

Introduction

Phytoalexins are low molecular mass compounds produced by higher plants in response to microbial infection or other forms of stress. These compounds can be microcidal or microstatic and play a key role in the plant defense system (Barz et al. 1990, Smith 1996). Phytoalexin production can be elicited by a number of biotic and abiotic factors. The phytoalexin 6-methoxymellin (8-hydroxy-3-methoxy-3,4-dihydro-isocoumarin; 6-ME) (Sondheimer 1957) is produced in carrot roots following pathogen infection. 6-ME can also be induced by ethylene (Chalutz et al. 1969, Lafuente et al. 1996), heavy metals (Marinelli et al. 1991), UV irradiation (Yates 1987, Mercier et al. 1993), buthionine sulfoximine (Guo et al. 1993), 2,4-dichlorophenoxyacetic acid (2,4-D) (Chalutz et al. 1969, Kurosaki et al. 1985a), polysaccharides derived from carrot cell walls (Kurosaki and Nishi 1983), and lytic enzymes that degrade plant cell walls (Kurosaki et al., 1985b, Amin et al. 1986). Accumulation of 6-ME by UV light has been studied as a means to reduce carrot decay (Mercier et al. 1993). The ethylene synthesis inhibitor, aminoethoxyvinylglycine (AVG), reduces but does not completely inhibit accumulation of 6-ME induced by yeast glycan, suggesting there is an ethylene-independent pathway for synthesis of 6-ME (Guo and Ohta 1994). To further examine the role of ethylene in phytoalexin synthesis, the ethylene action inhibitor 1-methylcyclopropene (MCP) (Sisler and Blankenship 1996, Sisler and Serek 1997) was used in this study to treat carrot roots prior to the use of elicitors of 6-ME production.

Materials and methods

Plant source

Carrot (*Daucus carota* L.) roots were obtained from a local farm or market and used either immediately or stored at 0°C prior to use.

Chemicals

Ethylbloc was obtained from Floralife (Walterboro, SC, USA). All other chemicals were obtained from Sigma (St Louis, MO, USA).

MCP treatments

Carrots were surface sterilized in a 0.01% NaOCl solution for 2 min, rinsed twice with distilled water, then exposed to 1 μl l^{-1} MCP or air FOR 4 h in sealed 20-l glass jars at 10°C. To generate MCP, Ethylbloc powder was placed inside an 18 × 150 mm test tube, then the tube was capped with a serum stopper. MCP gas was generated by injecting buffer solution supplied by the manufacturer through the serum stopper using a syringe with a 20 gauge needle. A tube containing MCP gas was placed inside the neck of the 20-l glass jar containing carrots, then the jar was sealed using flexible polyethylene (Saran Wrap) secured with rubber bands. The tube was uncovered by dislodging the septum cap through the wrap, and then another layer of wrap was added to assure a tight seal.

Abbreviations – AOA, aminooxyacetic acid; AVG, aminoethoxyvinylglycine; 6-ME, 6-methoxymellin; MJ, methyl jasmonate; PDA, potato dextrose agar.

MCP concentration in the jars was analyzed at the end of the 4-h treatment period by removing a 0.5-ml gas sample through the wrap seal using a tuberculin syringe. The sample was analyzed by gas chromatography using a HP 5880 GC (Hewlett Packard, Avondale, PA, USA) and 1-butene as an external standard. The GC column (60 cm stainless steel, 2 mm i.d.) was packed with 80/100 mesh Porapak Q (Alltech Assoc. Inc., Deerfield, IL, USA). Injector, oven and flame ionization detector temperatures were 100, 130 and 200°C, respectively. Gas flows for N_2 carrier, H_2 and air were 30, 30 and 300 ml min^{-1}, respectively.

Ethylene-induced 6-ME accumulation

After MCP treatment, 4 replicate (approximately 400 g each) carrot samples were placed into 4-l glass jars at 10°C with air or continuous 1 μl l^{-1} ethylene in air flowing at 5 l h^{-1}. There were 4 treatments: air/air, air/μl l^{-1} ethylene, 1 μl l^{-1} MCP/air and 1 μl l^{-1} MCP/1 μl l^{-1} ethylene. Peel (3 g) and pulp (6 g) (cortex plus vascular tissues) samples were collected initially and 2, 4, 8, 12, and 16 days after initiation of the ethylene treatment. Tissue samples were frozen in liquid nitrogen, then stored at −20°C prior to analysis according to Sondheimer (1957) and Lafuente et al. (1996). Extraction of 6-ME was performed for 12 h at ambient temperature using spectrophotometric grade hexane. The solution was decanted after extraction and then re-extracted with an equal volume of 80% ethanol. Absorbance of the ethanol layer was measured at 267 nm and 6-ME was calculated using a molar absorptivity of 14800.

Pathogen-induced 6-ME production

MCP-treated carrots were surface sterilized a second time by spraying with an 80% ethanol solution. The carrots were rinsed with sterilized water and air-dried in a laminar-flow hood. Discs (approximately 2 cm diameter, 3–4 mm thickness) were prepared from uniform carrots with a sterilized razor blade. Discs were placed on moist filter paper in Petri dishes, and a 10 μl aliquot of water (controls) or a conidial suspension (10^5 conidia ml^{-1} H_2O) was applied onto the center of each disc. Conidia from 3 fungi were used: *Alternaria brassicicola* (Schweinitz) Wiltshire, *Alternaria radicina* Meier Drechsler & Eddy, and *Penicillium expansum* Link ex. Gray. Conidia were obtained from fungi grown on PDA media for 7–14 days. Each Petri dish contained 5 carrot discs and each dish was treated as one replicate. There were 4 replicates for each treatment. The discs were incubated at 20°C. At 0, 2 or 4 days after incubation, the discs were quartered, 5 g randomized samples were extracted with hexane and used for 6-ME determination.

2,4-D- and MJ-induced 6-ME production

Discs were prepared as described previously treated from MCP-treated carrots. The discs were immersed in 100 μl l^{-1} 2,4-D or 1 m*M* methyl jasmonate (MJ) for 1 min, then placed on Petri dishes and incubated at 20°C. There were 4 replicates and 5 discs per replicate. Analysis of 6-ME in 5 g samples was performed after incubation at 20°C for 0, 2, or 4 days.

UV-B-induced 6-ME production

The surface of whole carrot roots was rubbed with steel wool to enhance the response to UV irradiation (Mercier et al. 1993). The carrots were then surface sterilized by immersion in a 0.01% NaOCl solution for 2 min, then rinsed twice with distilled water. Carrots were then treated with 1 μl l^{-1} MCP or air for 12 h in sealed 20-l glass jars at 3°C. Following MCP treatment, carrots were placed under UV-B light (1.80 W m^{-2} at 310 nm) for 7 days at 3°C. There were 3 replicates with 5 carrots per replicate. Peel samples (3 g) were prepared from UV-B-exposed carrots and analyzed for 6-ME as previously described.

All experiments were repeated at least twice and results were similar. Therefore, only representative results are presented. Least significant difference (LSD) values ($\rho \leq 0.05$) and standard deviation (SD) were calculated using SAS (SAS Institute, Cary, NC, USA).

Results

The 6-ME content of control and MCP-treated carrots remained constant in both peel and pulp tissues during 16 days at 10°C (Fig. 1). Peel 6-ME content was 6-fold higher than pulp as previously reported (Lafuente et al. 1996). Ethylene exposure resulted in a significant accumulation of 6-ME in both peel and pulp, 25- and 33-fold increases, respectively, compared to controls after 12 days. Peel accumulated about 3-fold more 6-ME than pulp after exposure to ethylene for 12 days. MCP treatment prior to ethylene exposure totally inhibited the ethylene-induced 6-ME accumulation.

Accumulation of 6-ME in carrot discs varied with the pathogen tested (Fig. 2). *A. radicina* was the most effective elicitor of 6-ME accumulation followed by *P. expansum* and *A. brassicicola*. Exposure to MCP before inoculation inhibited 6-ME accumulation. *A. radicina* grew extensively on carrot tissues while *A. brassicicola* and *P. expansum* grew poorly (data not shown). *A. radicina* also grew faster on discs prepared from MCP-treated carrots than on discs from controls (data not shown).

2,4-D induced 6-ME accumulation during the 4-day post-treatment incubation period at 20°C (Fig. 3). The 6-ME content in 2,4-D-treated carrot discs was more than 14-fold higher than the amount in control discs. MCP treatment inhibited 6-ME accumulation in the presence of 2,4-D. MJ treatment also resulted in increased 6-ME content, but accumulation was lower than that induced by 2,4-D. Exposure to MCP totally eliminated the increase in 6-ME content stimulated by MJ.

UV-B irradiation induced significant accumulation of 6-ME. After 7 days at 3°C, the peel of control carrots had 0.90 mg (100 g fresh weight)$^{-1}$ 6-ME, peels of MCP-treated carrots accumulated 0.58 mg (100 g fresh weight)$^{-1}$ 6-ME and UV-B-treated carrots had 11.91 mg (100 g fresh weight)$^{-1}$ 6-ME, significantly (LSD, $\rho = 0.05$) higher than

that in control carrots. Exposure to MCP before UV-B treatment reduced the 6-ME content to 1.69 mg (100 g fresh weight)$^{-1}$.

Discussion

MCP is an ethylene action inhibitor (Sisler and Blankenship 1996) that prevents a variety of ethylene-mediated responses in plants (Sisler and Serek 1997). The lack of 6-ME accumulation in response to ethylene, UV-B radiation, 2,4-D, methyl jasmonate, or inoculation with *A. brassicicola*, *A. radicina* or *P. expansum* following MCP treatment of carrot roots indicates these elicitors induce 6-ME through the ethylene signaling pathway. Cutting whole carrots previously treated with MCP did not reduce the inhibitory effect of MCP, indicating that wounding does not generate new ethylene receptors that are part of the same signaling pathway. Pathogen attack can induce enzymatic degradation of cell walls resulting in accumulation of polysaccharides that elicit production of phytoalexins including 6-ME (Kurosaki and Nishi 1986). The variation in ability to induce 6-ME by fungi may be caused by differences in enzyme production and/or the ability of different fungi to grow on carrot discs.

Production of ethylene induced by pathogens is correlated with the accumulation of phytoalexin (Chalutz et al. 1969, Guo et al. 1993). 2,4-D (Chalutz et al. 1969, Kurosaki et al. 1985a), MJ (Sanz et al. 1993, Fan et al. 1998), and UV (Predieri et al. 1995) also induce ethylene biosynthesis. Exposure to MJ or UV also induce synthesis of proteinase inhibitors (Farmer et al. 1992, Conconi et al. 1996) and other proteins (Penninckx et al. 1998) that function in plant defense against pathogens. Induction of proteinase inhibitors and other proteins is mediated in part through ethylene (O'Donnell et al. 1996, Thomma et al. 1999).

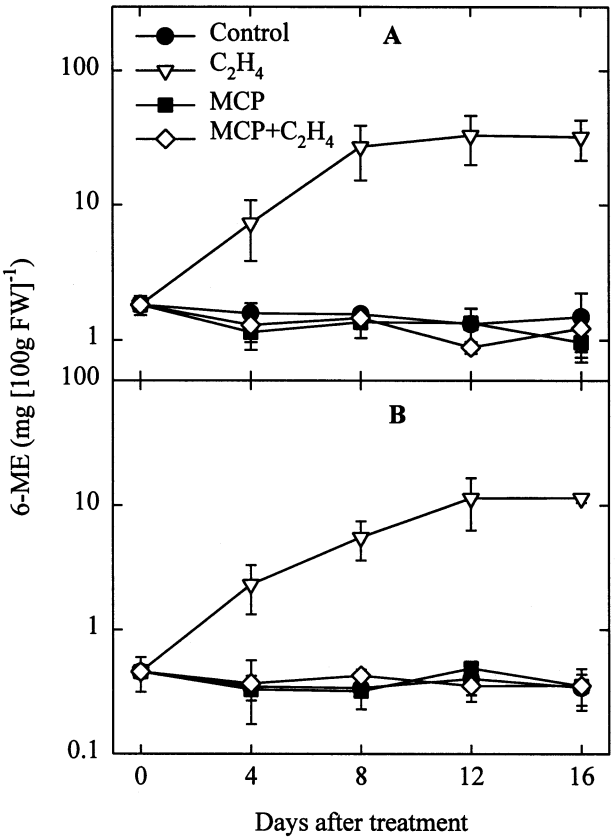

Fig. 1. Interactive effects of ethylene and MCP on accumulation of 6-ME in carrot peel (A) and pulp (B). Whole carrots were treated with air (control) or 1 µl l^{-1} MCP for 4 h then continuously exposed to air or 1 µl l^{-1} ethylene at 10°C. Vertical bars represent SD of 4 replicates.

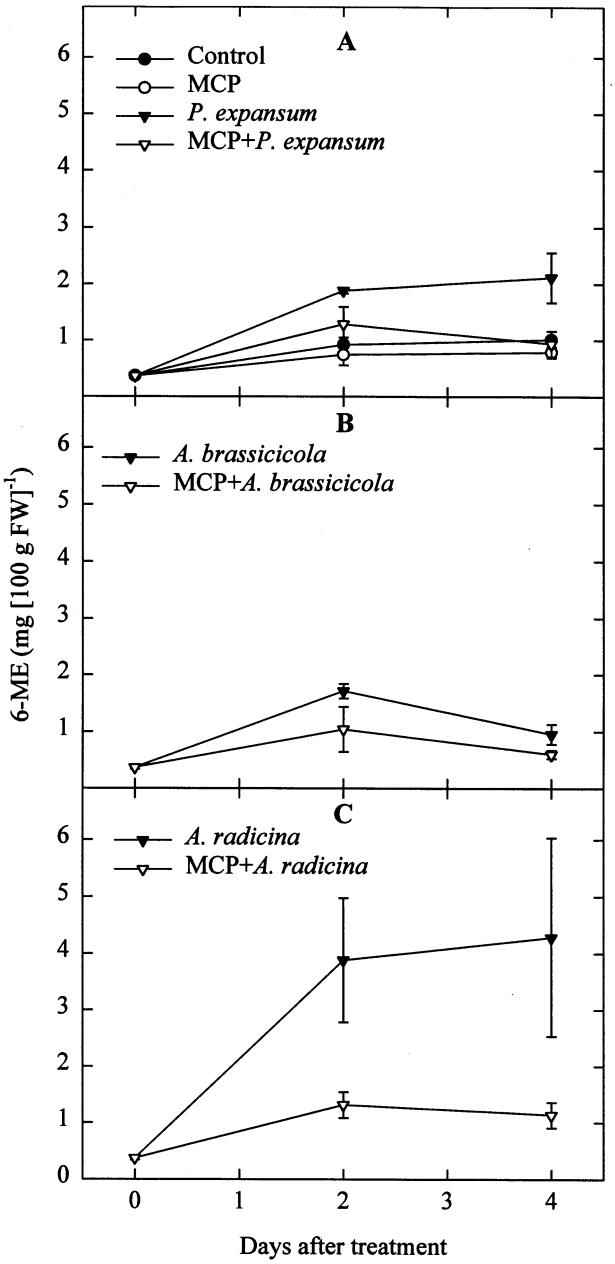

Fig. 2. Interactive effects of pathogen inoculation and MCP on accumulation of 6-ME in carrot discs. Whole carrots were treated with air (control) or 1 µl l^{-1} MCP for 4 h, then discs were inoculated with water or *P. expansum* (A), *A. brassicicola* (B), and *A. radicina* (C), and incubated at 20°C. Vertical bars represent SD of 4 replicates.

Although a role for ethylene in stimulating plant defense responses to pathogen attack has been demonstrated (Boller 1991), ethylene does not enhance resistance to all pathogens. Ethylene can actually increase or decrease plant susceptibility to pathogens (Lund et al. 1998). The ethylene signal transduction pathway is composed of a family of receptors (Bleeker and Schaller 1996, Ecker 1995). Mutations in these receptors can confer ethylene insensitivity. For example, expression of pathogen-induced plant defense genes (i.e. for chitinase and defensin) is nearly completely abolished in the *ein2-1* mutant. The *ein2-1* mutant is more susceptible to *Botrytis cinerea* compared to wild-type plants, but susceptibility to *A. brassicicola* is not altered (Thomma et al. 1999). The ethylene insensitive tomato mutant *Never Ripe* exhibits increased susceptibility to some pathogens while susceptibility to others is reduced (Lund et al. 1998). Ethylene mediated responses may play a role in host resistance to some but not all types of pathogens depending on the mode of pathogenicity specific to each pathogen. Other response pathways such as those mediated by jasmonate are also required for induction of some plant defense genes (Xu et al. 1994, Penninckx et al., 1998). Our results indicate that inhibition of ethylene action decreases phytoalexin accumulation; however, production of phytoalexin is only one part of the total response to pathogen attack. Other defense mechanisms include an oxidative burst (Doke et al. 1996, Low and Meroda 1996, Wojtaszek 1997), reinforcement of cell walls by deposition of lignin, polysaccharides and structural proteins; and the accumulation of defense related proteins (Boller 1991). Ethylene induces synthesis of hydroxyproline-rich glycoproteins associated with cell walls (Esquerre-Tugaye et al. 1979). The presence of these proteins is positively correlated with pathogen resistance (Esquerre-Tugaye et al. 1979), and activates antifungal hydrolases such as chitinase and glucanases (Ecker and Davis 1987, Harber and Fuchigami 1989, Kurosaki et al. 1989, Boller 1991).

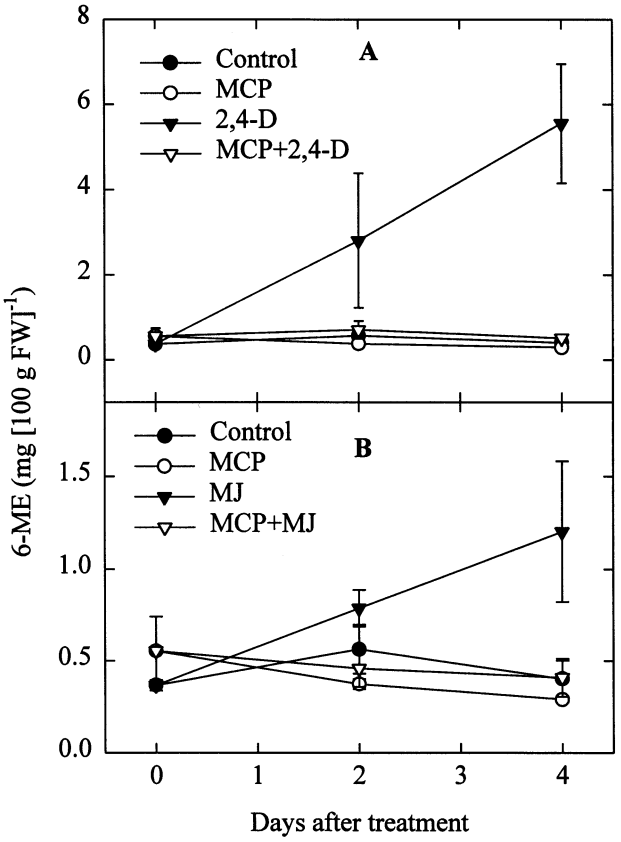

Fig. 3. Interactive effects of 2,4-D (A) or MJ (B) with MCP on accumulation of 6-ME in carrot discs. Whole carrots were treated with air (control) or 1 μl l^{-1} MCP for 4 h then discs were treated with water or 100 μl l^{-1} 2,4-D and 1 m*M* MJ, and incubated at 20°C. Vertical bars represent SD of 4 replicates.

The presence of 6-ME effectively inhibits pathogen growth (Kurosaki and Nishi 1983, Mercier et al. 1993). Inhibition of 6-ME synthesis after *A. radicina* inoculation of discs prepared from MCP-treated carrots may have allowed pathogen growth to exceed the growth rate on inoculated control discs (data not shown). Inhibition of ethylene action by MCP promotes decay in citrus (Porat et al. 1999). Citrus produces coumarins (6,7-dimethoxycoumarin and 7-hydroxy-6-methoxycoumarin) in response to pathogen attack, and these phytoalexins may be resistance factors (Afek and Sztejnberg 1988). Resistant citrus species, treated with the ethylene synthesis inhibitor AOA, become susceptible to *Phytophthora citrophthora* (Afek and Sztejnberg 1988). In these cases, MCP-promoted decay may result from the lack of phytoalexin accumulation (Fig. 2).

Synthesis of 6-ME occurs via the polyketide pathway through condensation of one acetyl- and 4 malonyl-CoAs (Kurosaki and Nishi 1988, Kurosaki 1994). Many other phytoalexins are synthesized from the phenylpropanoid pathway. Ethylene (Boller 1991) and some elicitors of phytoalexin production, including MJ (Bleschert et al. 1995, Milksch and Boland 1996) and UV (Gleitz et al. 1991, Glassgen et al. 1998), have been shown to induce phenylalanine ammonia lyase and other enzymes in this pathway. Whether accumulation of phytoalexin from the phenylpropanoid pathway is mediated through ethylene action is currently unknown.

Production of 6-ME in carrot is activated by ethylene, pathogen infection, UV irradiation, 2,4-D, and MJ. The production of 6-ME can be inhibited by blocking ethylene action using MCP, indicating that the biosynthesis of this phytoalexin in carrot tissue is ethylene-mediated. These results confirm the important role of ethylene in mediating defense responses of plants to microbial pathogens.

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